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Jeanine Enewold Goldberg
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Mailbox-- 12E12
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Biologic Activity of Mitochondrial Metabolites on Aging and Age-Related Hearing Loss

*Michael D. Seidman, *Mumtaz J. Khan, *Uma Bai, *Najeeb Shirwany, and
†Wayne S. Quirk

*Department of Otolaryngology Head & Neck Surgery, Henry Ford Health System, Detroit, Michigan,
and †Minnesota State University, Mankato, Minnesota, U.S.A.

Hypothesis: Compounds that upregulate mitochondrial function in an aging model will improve hearing and reduce some of the effects of aging.

Background: Reactive oxygen metabolites (ROM) are known products of oxidative metabolism and are continuously generated in vivo. More than 100 human clinical conditions have been associated with ROM, including atherosclerosis, arthritis, autoimmune diseases, cancers, heart disease, cerebrovascular accidents, and aging. The ROM are extremely reactive and cause extensive DNA, cellular, and tissue damage. Specific deletions within the mitochondrial DNA (mtDNA) occur with increasing frequency in age and presbycusis. These deletions are the result of chronic exposure to ROM. When enough mtDNA damage accrues, the cell becomes bioenergetically deficient. This mechanism is the basis of the mitochondrial clock theory of aging, also known as the membrane hypothesis of aging. Nutritional compounds have been identified that enhance mitochondrial function and reverse several age-related processes. It is the purpose of this article to describe the effects of two mitochondrial metabolites, α -lipoic acid and acetyl L-carnitine, on the preservation of age-related hearing loss.

Methods: Twenty-one Fischer rats, aged 24 months, were divided into three groups: acetyl-L-carnitine, α -lipoic acid, and control. The subjects were orally supplemented with either a

placebo or one of the two nutritional compounds for 6 weeks. Auditory brainstem response testing was used to obtain baseline and posttreatment hearing thresholds. Cochlear, brain, and skeletal muscle tissues were obtained to assess for mtDNA mutations.

Results: The control group demonstrated an expected age-associated threshold deterioration of 3 to 7 dB in the 6-week study. The treated subjects experienced a delay in progression of hearing loss. Acetyl-L-carnitine improved auditory thresholds during the same time period ($p < 0.05$). The mtDNA deletions associated with aging and presbycusis were reduced in the treated groups in comparison with controls.

Conclusions: These results indicate that in the proposed decline in mitochondrial function with age, senescence may be delayed by treatment with mitochondrial metabolites. Acetyl-L-carnitine and α -lipoic acid reduce age-associated deterioration in auditory sensitivity and improve cochlear function. This effect appears to be related to the mitochondrial metabolite ability to protect and repair age-induced cochlear mtDNA damage, thereby upregulating mitochondrial function and improving energy-producing capabilities. **Key Words:** Aging—Age-related hearing loss—Mitochondrial function—Acetyl-L-carnitine— α -Lipoic acid.

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Presbycusis, the progressive deterioration of hearing associated with aging, is the most common cause of hearing loss in the United States. Twenty-three percent of the population between ages 65 and 75, and 40% of the population over age 75, are reported to be affected (1). Presbycusis results in a progressive bilateral high-frequency auditory loss, which has significant socioeconomic implications.

In recent years, the effects of reactive oxygen metabolites (ROM), also known as free radicals, and their me-

tabolites on biologic systems have received much attention. These ROM are known to play important roles in many biochemical reactions that maintain normal cell functions. Increasing evidence indicates that ROM are also important mediators of several forms of tissue damage, such as injuries associated with inflammatory responses, ischemic injuries to organs, and injuries resulting from the intracellular metabolism of chemicals and drugs. The ROM are increasingly recognized for their contribution to tissue injury during ischemia and, in particular, during the phase of reperfusion and prolonged hypoperfusion. The primary in vivo source of ROM appears to be the mitochondrial electron transport system during oxidative phosphorylation. Other sources of ROM include purine catabolism by xanthine oxidase, prostaglandin biosynthesis, infiltration of phagocytes, environ-

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Address correspondence and reprint requests to Dr. Michael D. Seidman, Henry Ford Health System, Department of Oto-HNS, 6777 W. Maple Rd., W. Bloomfield, Michigan 48323.

mental contaminants, ionizing radiation, and aging. Many components within the cell are susceptible to attack by ROM.

The generation of ROM occurs from periods of prolonged relative hypoperfusion, such as that occurring with aging. It has been demonstrated that in the elderly there is significantly decreased flow within the circulatory system in general (2-5) and the inner ear specifically (6,7). Prolonged periods of reduced blood flow, such as those accompanying aging, lead to the formation of tissue-damaging ROM. The ROM have been implicated as mediators in mitochondrial DNA damage, including the generation of mitochondrial DNA deletions (mtDNA del), which have been associated with cellular and tissue dysfunction, senescence, and death. This sequence of events is the foundation of the membrane hypothesis of aging (7).

The mtDNA genome is a circular molecule consisting of 16,569 base pairs (bp). The mitochondria are responsible for >90% of the cellular energy production, and the primary byproduct of energy metabolism is ROM generation. The ROM cause significant structural assault on the mitochondrial DNA genome. This damage is demonstrated by insertion mutations, deletions, and other forms of damage to the mtDNA. One specific deletion that occurs secondary to ischemia, aging, and even presbycusis is known as the common human aging deletion and involves a 4977-bp segment of the mtDNA (8-10). The 4977-bp mtDNA del leads to a reduced ability of the mitochondria to produce energy. This is manifested by reductions in mitochondrial membrane potentials, a measure of mitochondrial function and reduced overall energy production. Additionally, reductions in oxidative phosphorylation have been identified. A threshold exists when enough deletions occur so that the cell becomes bioenergetically deficient.

MITOCHONDRIAL METABOLITES

Acetyl-L-carnitine

Acetyl-L-carnitine (ALCAR) is the acetyl ester of carnitine, a biologic compound that plays a key role in the transport of fatty acids from the cytosol into the mitochondrial matrix for B-oxidation. This serves as a key source of energy for many tissues. The activity of carnitine-acylcarnitine exchange across the inner mitochondrial membrane is of great importance for energy production. Investigation of heart mitochondria indicates that the activity of this exchange reaction decreases significantly with age (11). The ALCAR modulates, through regulation of acetyl-CoA, the metabolism of sugars, lipids, and amino acids, thereby playing a pivotal role in cellular energy and turnover of cell membranes and proteins. Long-term treatment with ALCAR enhances stimulation of antiperoxidative systems, antagonism of the age-related effect on glucocorticoid secretion (12), increase in acetylcholine release (13), and improvement in learning and memory (14,15). The multiplicative effects of ALCAR in reversing the age-related decline in

various physiologic parameters associated with mitochondrial function may be attributable to its ability to deliver acetyl-CoA equivalents to the tricarboxylic acid cycle and to facilitate the mitochondrial B-oxidation of fatty acids, thereby increasing the production of ATP.

α -Lipoic acid

α -Lipoic acid is a coenzyme for the pyruvate dehydrogenase complex in the mitochondrial matrix. It is an essential cofactor for metabolism in α -ketoacid dehydrogenase reactions. In physiologic systems, α -lipoic acid usually exists as lipoamide covalently attached to the lysine residue of the enzyme complex. It functions in the transfer of the two-carbon fragment from α -hydroxyethylthiamin pyrophosphate to acetyl-CoA, and itself gets reduced in the process. The reduced form of α -lipoic acid is dihydrolipoic acid containing a disulfhydryl structure. Dihydrolipoic acid is the active form possessing antioxidant properties. It has been demonstrated to prevent microsomal lipid peroxidation by converting glutathione to its reduced form, which in turn recycles vitamin E (16). Dihydrolipoic acid has also been demonstrated to be a reactive oxygen metabolite scavenger (17); to reduce peroxy, ascorbyl, and chromanoxyl radicals (18); and to inhibit singlet oxygen (19).

Several studies have demonstrated the ability of these mitochondrial metabolites to enhance mitochondrial membrane potentials and energy production (20). Thus, it is the purpose of this article to discuss the effects of mitochondrial metabolites on age-associated hearing loss and mtDNA deletions. To our best knowledge, this study is the first to report the effects of these compounds on the auditory system.

METHODS

Subjects

Fischer 344 rats, aged 24 months, purchased from the National Institute of Aging, served as the experimental subjects. The animals were maintained at 21° to 22°C in group cages under a 12:12-hour light-dark cycle initiated at 0700 hour. All experiments were reviewed and approved by the Henry Ford Health System Care for Experimental Animal Committee. Animal care protocols were in strict compliance with established guidelines of the National Institutes of Health.

Mitochondrial metabolites

The mitochondrial metabolites α -lipoic acid and acetyl L-carnitine were obtained from Weinstein Pharmaceuticals, Anaheim, CA, U.S.A. These substances have been used for human and rodent studies and have not shown any side effects at the desired dosages.

Protocol

To investigate the effects of mitochondrial metabolites on hearing and mitochondrial function, animals were randomly divided into three groups ($n = 7$ for each group). Each subject was housed individually in metabolic cages and had its diet supplemented with one of the following substances:

- Group 1: α -lipoic acid (300 mg/kg/day) ($n = 7$)
- Group 2: acetyl-L-carnitine (300 mg/kg/day) ($n = 7$)

A third group, the control group, received a regular diet without any supplementation.

Baseline levels of auditory sensitivity were obtained for each subject using auditory brainstem responses (ABR). After 6 weeks of supplementation, repeat ABR were obtained to assess for any changes in auditory thresholds. At the conclusion of the hearing threshold measurements, skeletal muscle, brain, liver and cochlear tissue including stria vascularis and auditory nerve were obtained for mtDNA analysis and to determine the presence of mtDNA del using the polymerase chain reaction. In particular, the 4834-bp mtDNA del associated with aging and presbycusis in rodents (10) were studied and quantified. This aging deletion seen in rodents corresponds with the 4977-bp deletion seen in humans.

Auditory brainstem responses

ABR testing was performed at the beginning and end of the study, 6 weeks after the initial ABR test was performed. The animals were anesthetized with Ketaset and Rompun (100 mg/kg and 15 mg/kg, respectively, intramuscularly) with Ketaset supplementation as required. The subject's head was secured in a head holder, and temperature was maintained with a thermostatically controlled heating blanket and rectal probe. A Bruel & Kjaer (Germany) condenser microphone with speculum was placed in the external auditory canal and held 2 to 3 mm from the tympanic membrane. Sterile 1/2" 26-gauge needles were placed under each pinna and at the vertex. Wires from each needle electrode were led to a Grass (Quincy, MA, U.S.A.) P511H amplifier, gain $\times 5000$ (band pass of 0.3 to 3.0 kHz) and then to a signal processing board (Spectrum [Vancouver, Canada], Model TMS320C25). The output of the biologic amplifier was viewed on an oscilloscope (Tektronics [Beaverton, OR, U.S.A.], Model 5112). The average waveforms were stored on a Pentium computer for offline analysis. A total of 512 samples, 25-microsecond bin width, 256 responses were averaged. Tone bursts (1.0 millisecond rise-fall time, 15 millisecond duration) were used to assess the auditory sensitivity. Intensity series were obtained at 3.0, 6.0, 9.0, 12.0, and 18 kHz. The waveforms were recorded and saved for offline analysis.

DNA extraction

Tissue samples were obtained and stored at -70°C until the time of DNA extraction. The tissue samples were homogenized in 10 mM Tris (pH 8.0) containing 1 mM EDTA buffer and incubated overnight at -56°C with 15 μl proteinase-K (10 mg/ml) in 0.5 ml digestion buffer consisting of 10 mM Tris (pH 8.0), 10 mM EDTA, 50 mM NaCl, and 2% sodium dodecyl sulfate. Standard extraction protocols for DNA were used with phenol, chloroform, and isoamyl alcohol. The proteins were removed from the sample solution with phenol:chloroform (25:24), both of which served as separate organic solvents and hence deproteinized more efficiently. The tissue extracts were

then centrifuged at $10,000 \times g$ at room temperature to separate mtDNA from cellular debris, proteins, and genomic DNA. The supernatant was drawn off, and the residual phenol was removed with equal volumes of chloroform:isoamyl alcohol (24:1). This subsequent extraction with chloroform removed the remaining traces of phenol from the preparation. Then, 1/10 volumes of 3M NaOAc and 1/100 volume of 1M MgCl_2 were added, and mtDNA was recovered by precipitation with 2.0 volumes of cold ethanol. This preparation was stored at -70°C for 60 minutes, and the precipitate was recovered by centrifugation at $12,000 \times g$ for 30 minutes (4°C). The supernatant was removed, and the pellet was washed with 70% ethanol, air-dried, and redissolved in TE buffer at the desired concentration. Polymerase chain reaction (PCR) was then performed on aliquots of this purified mtDNA (10).

Polymerase chain reaction

Each PCR reaction contained 150 ng of mtDNA from test sample, 200 mM of each dNTP, 50 mM KCL, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl_2 , 0.01% (wt/vol) gelatin, 1 mM of each primer, and 5.0 U of taq polymerase in a final volume of 100 μl . The thermal cycling parameters were as follows: initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C (30 seconds), annealing at 56°C (30 s) and extension at 72°C (60 seconds). Specific primers designed in our laboratory have been synthesized (by Operon Technologies, Alameda, CA, U.S.A.) to amplify distinct regions of the mtDNA genome (Table 1). These specific segments include the ND1-16S rRNA genome as well as the mtDNA⁴⁸³⁴ common aging deletion.

Gel electrophoresis

The amplified PCR products were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide. Gel electrophoresis was performed at 100 volts for 3 hours. Gels were then read under ultraviolet light and imaged.

DNA quantification

Quantitative PCR was performed with PCR ELISA (DIG Detection) kit following the manufacturer's instructions (Boehringer Mannheim, Germany).

External standards were prepared by gel-purifying the PCR products of ND1-16S rRNA and the common aging deletion as described above. The PCR was performed with standards and the different DNA samples at the exponential phase. The PCR products were labeled with digoxigenin, using DIG-UTP during PCR. An aliquot of the labeled PCR products (2–5:1) was then bound to the streptavidin-coated surface of a microtiter plate by the use of a biotin-labeled capture probe. This capture probe had to be designed to hybridize to an internal sequence of the PCR product. The bound DIG-labeled PCR products were

TABLE 1. Primer sequences for rat mitochondrial DNA

Genome	Sequence	bp
Rat ND1-16S rRNA		601
Forward primer	5'-GCCTATCGAGCTTGGTGATA-3'-1440	
Reverse primer	5'-TATCCTACCTTTGCACGGTC-3'-2033	
Rat aging deletion		598
Forward primer	5'-GCGAAGCTTAGAGCGTTAAC-3'-7701	
Reverse primer	5'-AGTGAGATAAGGAAGCCTGC-3'-13110	

then detected with an anti-DIG-peroxidase conjugate and the substrate ABTS. The colorimetric signal at 405 nm allowed quantitative determination of the amount of PCR product. The ratio of the deleted mtDNA to the total mtDNA was recorded and compared between the control and treated groups.

RESULTS

The animals ($N=21$) were randomized into three groups: (a) acetyl-L-carnitine treatment, (b) α -lipoic acid treatment, and (c) Control. Over the 6-week study, the control group underwent a deterioration of 3 to 7 dB in auditory sensitivity. The greatest reduction in hearing sensitivity occurred at 3 kHz with a 7 dB reduction, and the least amount of hearing loss occurred at 18 kHz with a 3-dB threshold shift. By contrast, the subjects treated with the α -lipoic acid experienced an overall delay in progression of hearing loss over the 6-week treatment period. This difference was statistically significant at 3 kHz only ($p < 0.05$), but a trend was observed at 6, 9, 12, and 18 kHz. The threshold shifts at these frequencies were not statistically significant according to analysis of variance (ANOVA) and a two-tailed t test. The chance of a type II statistical error was nullified by Bonferroni correction and by using an adequate sample size of $n = 7$ for each group. The subjects treated with acetyl-L-carnitine, by further contrast, showed an actual improvement in hearing at all but one test frequency. The threshold changes noted were statistically significant at all frequencies ($p < 0.05$) except at 3 kHz ($p = 0.09$). Once again, ANOVA and two-tailed t test were used for statistical significance. These data are summarized in Figure 1.

After the posttreatment ABR, the subjects were killed, and skeletal muscle, brain, liver, and cochlear tissues

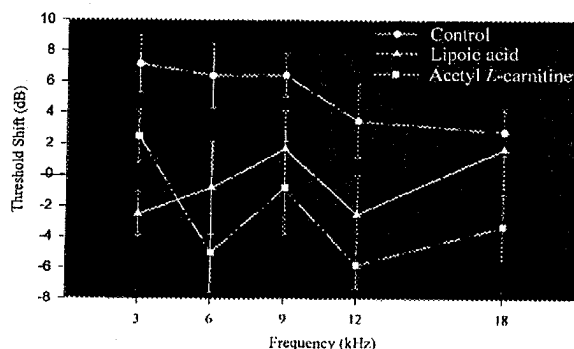


FIG. 1. Auditory threshold shifts in the three groups, as measured by auditory brain responses. Test frequencies are represented on the x axis and the threshold shifts on the y axis. Top slope demonstrates the shift in the control group. Lower slopes represent the effects of acetyl-L-carnitine (ALCAR) and α -lipoic acid on hearing loss. Error bars represent one standard deviation from the mean. The effect of ALCAR in delaying the progression of hearing loss was statistically significant at all test frequencies, except at 3 kHz.

were obtained. Specifically, mtDNA from brain, stria vascularis, and auditory nerve was studied. To verify the presence of mtDNA, we designed appropriate oligonucleotide sequences to identify the ND-1 16S rRNA segment, which is a highly preserved region of the mitochondrial genome. Specific primers for the common aging deletion were also synthesized to test for the presence of this deletion in the tissue samples. Equal quantities of mtDNA were used in all samples for standardization. The ND-1 6SrRNA region is identified by a 601bp product, and the common aging deletion (4834-bp deletion) is identified by a 598-bp product (Fig. 2), in which Gel A represents the amplification of the ND-1 16SrRNA region in both the control and the treated samples, confirming the presence of mtDNA, and Gel B shows the presence of the 4834-bp common aging deletion in the mitochondrial genome. This product was identified in the control and treated subjects as well. Because equal quantity of DNA was studied in all samples for standardization, qualitative analysis revealed that the common aging deletion was present to a lesser degree in either of the treatment groups (Fig. 2, Gel B). Quantitative evaluation confirmed these findings. Quantitative determination of the deletions revealed a reduction in the ratio of the 4834-bp deletion to the total mtDNA in both the subjects treated with α -lipoic acid and those treated with acetyl-L-carnitine (Fig. 3). The ratio of the deleted to total mtDNA was compared between each tissue type in all groups.

DISCUSSION

The data presented here provide evidence for a novel treatment that appears not only to reduce the gradual age-associated decline in hearing sensitivity in rats but also to reduce the quantity of mtDNA del in the treated groups, which in turn provides for enhanced mitochondrial function.

In the current study, the control group continued to lose auditory sensitivity over time, as expected. This amount of progressive hearing loss at 24 months of age has been previously demonstrated in our laboratory (7,10). In the α -lipoic acid group, ABR testing showed reduced threshold shift at all frequencies; however, statistical significance was not achieved except at 3 kHz ($p < 0.05$). By contrast, the acetyl-L-carnitine group showed a protective effect at all frequencies ($p < 0.05$) except 3 kHz, ($p = 0.09$). The mechanisms behind the beneficial effects of these metabolites become apparent after a discussion of some of the mechanisms of aging.

The foundation of these studies is conceived at a molecular level that considers the membrane hypothesis of aging, also known as the mitochondrial clock theory of aging, as the probable mechanism behind presbycusis. To have a clear understanding of the membrane hypothesis of aging, we shall discuss some of the basic properties involved in senescence.

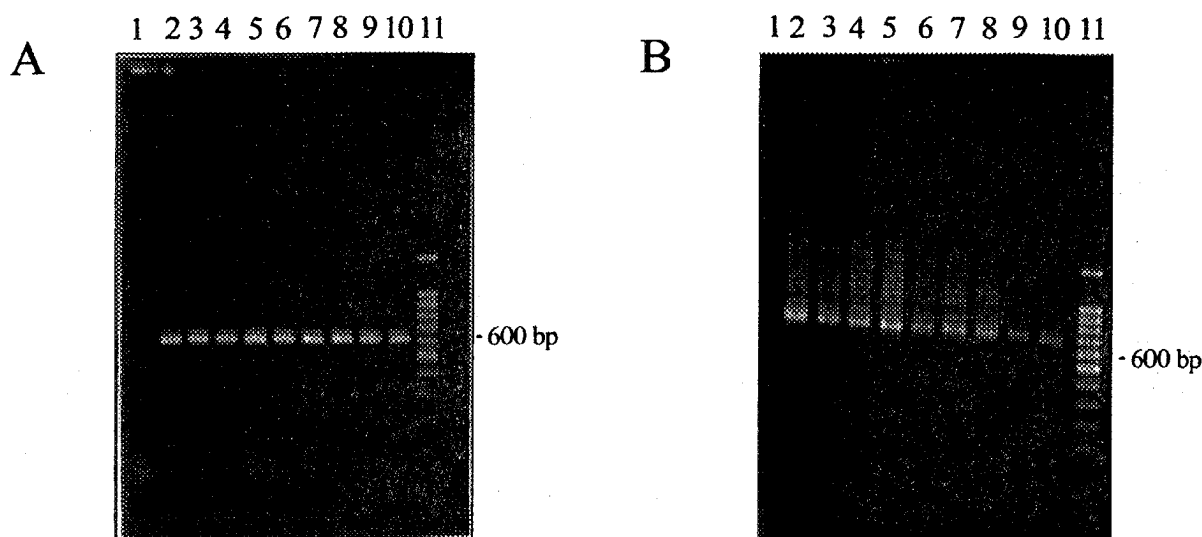


FIG. 2. Polymerase chain reaction. Gel A: Gel profile of ND1-16S rRNA amplified from tissue samples. Gel B: Gel profile of mtDNA⁴⁸³⁴ common aging deletion amplified from tissue samples. Lanes in both Gels A and B represent the following: 1. Control with no DNA. 2. Brain. 3. Auditory nerve (control group). 4. Stria vascularis. 5. Brain. 6. Auditory nerve (group treated with α -lipoic acid). 7. Stria vascularis. 8. Brain. 9. Auditory nerve (group treated with acetyl-L-carnitine). 10. Stria vascularis. 11. 100 bp ladder.

The process of aging is associated with many molecular, biochemical, and physiologic changes, including increases in DNA damage, reduction in mitochondrial function, decreases in cellular water concentrations, ionic changes, and decreased elasticity of cellular membranes. One contributing factor to this process is altered vascular characteristics, such as reduced flow and vascular plasticity as well as increased vascular permeability (7). These age-related changes may result in reductions in oxygen and nutrient delivery and also waste elimination (2-5). These physiologic inefficiencies favor the production of ROM. Additionally, there is support in the literature for age-associated reduction in endogenous enzymes that protect from ROM damage, including superoxide dismutase, catalase, and glutathione (21,22). Collectively, these changes enhance the generation of ROM,

which are responsible for producing mitochondrial DNA damage, including mitochondrial DNA deletions. Specific deletions are known to be directly proportional to aging, such as the common aging deletion in humans, which is 4977 bp in length (4834 bp in rats). When mtDNA del reach a certain level, the mitochondria become bioenergetically inefficient. Recent studies in our laboratory have shown that aged animals have reductions in auditory sensitivity with reductions in mitochondrial-associated function and increases in mtDNA deletions. These findings have been demonstrated with rat, mouse, and human lymphocytes and human archival temporal bones (10,23).

An increasing body of evidence supports the role of supplementation with compounds that can upregulate mitochondrial function. Specifically, we have called these compounds mitochondrial metabolites. Mitochondrial metabolites have many diverse functions, as outlined earlier in this article. The primary mechanisms involved in protection from aging appear to be multifactorial and would include their antioxidant properties, enhanced ATP production, increased efficiency of CNS receptors, and cell-membrane stability.

The apparent age-related deficits in mitochondrial function could be slowed or reversed by ALCAR, a normal component of the inner mitochondrial membrane that serves as a precursor of acetyl-CoA as well as the neurotransmitter acetylcholine. ALCAR has been shown to reverse the age-related decrease in the levels of the mitochondrial membrane phospholipid cardiolipin and the activity of the phosphate carrier in rat heart mitochondria (24). Furthermore, the age-associated decrease

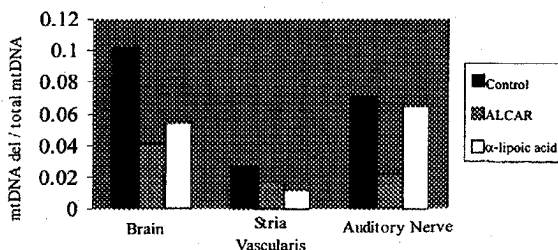


FIG. 3. MtDNA deletion quantification: Ratio of deleted mtDNA to the total mtDNA represented after quantification with enzyme-linked immunoassay (ELISA). Comparison of ratios in brain, auditory nerve, and stria vascularis made between the control group and the treated groups.

in mtDNA transcription is reversed rapidly by ALCAR. Aged rat brain and heart are reported to possess a reduced steady-state level of mitochondrial transcripts because of reduced RNA synthesis. Pretreatment of senescent rats with ALCAR restores the levels of mitochondrial transcripts to adult levels in a time- and dose-dependent function (20). The effects of ALCAR on mitochondrial function in the aging brain are supported by its ability to create a shift in ATP production from glycolytic pathways to the mitochondria (25). It is plausible that ALCAR can increase the metabolic efficiency of compromised subpopulations of mitochondria and cause a redistribution of the metabolic workload, resulting in increased cellular efficiency, and possibly decreases the rate at which mitochondria derived oxidants are produced.

α -Lipoic acid as an oral supplement is used for health benefits and has also been used as a therapeutic agent in a variety of hepatic and neurologic disorders as well as in mushroom poisoning. Consideration has also been given to the use of α -lipoic acid in the treatment of AIDS, atherosclerosis, and diabetes mellitus (17), in which decreased levels of α -lipoic acid have been found. Interestingly, a specific 10.4-kb mitochondrial DNA deletion has been found in patients with diabetes mellitus and sensorineural hearing loss. Thus, it may also be supposed that patients with these disorders might benefit from a diet supplemented with α -lipoic acid. Dietary supplementation of α -lipoic acid successfully prevents myocardial damage induced by ischemia-reperfusion injury (26). Presently, its primary therapeutic use is for the treatment of diabetic polyneuropathy (17).

Deafness has also been shown to have an association with mtDNA del. It has been suggested that mitochondrial diseases should be considered in cases of progressive sensorineural hearing loss, especially with the co-existence of multisystem involvement (27,28). Other studies have identified mutations in the tRNA-Leu gene in a large pedigree with maternally inherited diabetes mellitus type II and deafness (29). Several human studies have demonstrated an association of mitochondrial DNA mutations and presbycusis, including a study showing that older patients with presbycusis had a higher frequency of the common aging deletion (4977 bp) than did patients of similar age without presbycusis (Veda N, et al. Unpublished data). More recently, it has been demonstrated by use of human archival temporal bones that 14 of 17 aged patients with presbycusis had the 4977-bp deletion, compared with 8 of 17 control patients with normal hearing.

In conclusion, it is becoming increasingly clear that ROM production increases with aging. Concomitantly, there is a significant reduction in the antioxidant protective enzymes. The combined effect leads to an excess of oxidative damage, which causes mitochondrial mutations with reductions in the capacity for OXPHOS, hence reduced energy production. The current experiments provide a rationale to allow for improvement in this bioen-

ergetic deficiency by supplementing with mitochondrial metabolites.

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